Description of *Bursaphelenchus yongensis* sp.n. (Nematoda: Parasitaphelenchidae) isolated from *Pinus massoniana* in China

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Summary. *Bursaphelenchus yongensis* sp. n. isolated in Ningbo, China from *Pinus massoniana*, is described and illustrated. This nematode has a body length of 927 (816 - 1023) µm and 746 (699 - 926) µm for females and males, respectively, a slim body (a = 42 and 57, respectively), 13-14 µm long stylet, excretory pore at level of median bulb, lateral field with three lines, long post-uterine branch extending two thirds to three quarters of vulva to anus distance and a conoid female tail (c = 30) usually showing a 2-5 µm long mucron in central position at the terminus, male with small spicules (12-14 µm long) having distinct pointed rostrum, high distinctly dorsally bent condylus and small cucullus, and a dorso-ventrally visible terminal bursa of variable shape, but often two-pointed. *Bursaphelenchus yongensis* sp. n. differs morphologically from the most similar *B. eremus* by the presence of a cucullus at the distal end of the spicules, the differently-formed spicule condylus being higher and more strongly dorsally bent than in *B. eremus*, the presence of a small vulval flap and the longer mucron of females. The new species can be differentiated from *B. eremus* and 26 other *Bursaphelenchus* species by means of ITS-RFLP.

Key words: *Bursaphelenchus*, China, distribution, ITS-RFLP analysis, morphology, morphometrics, new species, Ningbo, Parasitaphelenchidae, taxonomy.

The pine wood nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 is the causal agent of pine wilt disease in East Asia, North America and Europe/Portugal (Evans *et al.*, 1996; Mota *et al.*, 1999). This nematode has been killing native pine trees (*Pinus densiflora*, *P. thunbergii*, *P. luchuensis*) in Japan since the early 20th century. It has been introduced into China (Cheng *et al.*, 1983) and has caused *Pinus massoniana* mortality in Ningbo since 1991 (Lai *et al.*, 1993). The local government has put large efforts in controlling the spreading of the disease.

During a continuing survey of the distribution of the pinewood nematode, *Bursaphelenchus xylophilus*, in China, other *Bursaphelenchus* species have also been detected. *Bursaphelenchus aberrans* Fang, Zhuo & Zhao, 2002 was found in dead wood of *Pinus massoniana* in Guangdong province, *B. hofmanni* Braasch, 1998 and *B. hellenicus* Skarmoutsos, Braasch & Michalopoulou, 1998 in *P. armandii* and *P. yunnanensis* in Yunnan province (Dang & Yu, 2003), and *B. hylobianum* Korenchenko, 1980 in *P. massoniana* in Fujian province (Wang *et al.*, 2004). *Bursaphelenchus lini* Braasch, 2004 was found in damaged and dead *P. thunbergii* and *P. massoniana* in Jiansu province (Braasch, 2004).

During a survey in Ningbo in 2004, *B. lini* was also found in dead *Pinus massoniana* trees in Caiqiao, Ningbo, and a previously undescribed

Bursaphelenchus species was found in Xiaogang, Ningbo. The morphological features, ITS-RFLP patterns and a description of this new species are presented in this paper.

MATERIAL AND METHODS

Branches from a dying pine tree of *Pinus massoniana* originating in Xiaogang, Ningbo, China were sawn into about 10 cm long pieces and subsequently cut into smaller pieces of no more than 1 cm width. Nematodes were extracted by the modified Baermann funnel technique for 48 h at 25°C and their morphology was studied using an Olympus BX50 microscope, fitted with a Furi CCD camera and with a Zeiss Axioskop 40 microscope. *Bursaphelenchus xylophilus* was not found in the samples. The new species multiplied on *Botrytis cinerea* growing on malt agar. A multiple specimen isolate culture was established. Adults were collected from a 3-week-old culture, heat-killed and fixed in FA 4:1, processed through a glycerol-ethanol series and mounted in glycerol according to Kleynhans (1999).

The *Bursaphelenchus* species used for molecular comparison were obtained as follows: *B. eremus* Rühm, 1956, which is most similar to the new species, was isolated from the vector *Scolytus intricatus* hatched from thin oak tree branches left on the ground in Eichhorst, Brandenburg, Germany, after felling the trees in 2004. The other isolates of *Bursaphelenchus* species used for molecular comparison originated in Germany (*B. hofmanni* Braasch, 1998, *B. pinasteri* Baujard, 1980), Austria (*B. leoni* Baujard, 1980) and China (*B. xylophilus*).

SEM micrographs were obtained with a scanning electron microscope Zeiss DSM 940 with cryotrans system Oxford CT 1500, using the analysis software 'analySIS 3.2'. Single *Bursaphelenchus* specimens were applied with a pipette onto a round piece of cellulose nitrate filter (8-µm pore-diameter) placed on a moist filter paper in a Petri dish. Immediately after application of the nematodes, the filter was fixed on a carbon tab and surrounded with disc emulsion of carbon on the specimen holder. It was then dipped into liquid nitrogen for rapid freezing as the first step for cryopreservation. Using a vacuum transfer device to prevent ice formation, the frozen specimens were transferred to the preparation chamber (for sputter coating and freeze fracture). Then, the nematodes were transferred to the microscope chamber for evaporation of water or ice under constant conditions (at temperatures increasing from −120° to -90°C under vacuum). Afterwards, the specimens were returned to the preparation chamber, which was filled with argon, and were coated for approx. 2.5 min with a thin film of argentum-palladium target; this process prevented the specimens from charging, avoided structural changes and ensured that they would have good conductivity. The two chambers (preparation and microscope) were cooled continuously by liquid nitrogen.

Nematode DNA was extracted from samples containing one to about 20 nematode specimens and isolated using the QIAamp DNA Micro Kit (Qiagen) as described by Burgermeister *et al.* (2005). In this procedure, DNA is purified by reversible adsorption to a silica matrix. DNA concentration of samples obtained from more than one specimen was determined fluorimetrically using a DyNa Quant 200 fluorometer (Amersham Biosciences) and the fluorescent dye, Hoe 33258.

For ITS-RFLP analysis, a segment of nematode ribosomal DNA containing the ITS-1 and ITS-2 regions was amplified by PCR, as described by Burgermeister *et al.* (2005). Suitable aliquots of the amplified DNA were digested with 3 units of the restriction endonucleases *Alu*I, *Hae*III, *Hinf*I, *Msp*I and *Rsa*I following the manufacturer's instructions. Restriction fragments were resolved by electrophoresis in a 2.5% agarose gel and stained with ethidium bromide.

DESCRIPTION

Bursaphelenchus yongensis sp. n. (Figs. 1 - 4)

Measurements (Table 1).

Female. Body cylindrical and slim, tapering at both ends. Heat relaxed form slightly ventrally arcuate. Lip region convex, 2.5-3 µm high, 7-8 µm wide, and offset by a distinct constriction. Stylet slender with very small basal swellings and with the shaft constituting about 65 % of total stylet length. Procorpus cylindrical. Median bulb oval and well developed. Valve plates in the middle of the median bulb or slightly behind the middle. Oesophageal gland lobe overlapping intestine dorsally for the length of three to five body widths. Excretory pore position at the level of median bulb. Nerve ring and intestine closely behind the median bulb. Reproductive system prodelphic, gonad outstretched, occupying about two thirds of the body length. Oocytes arranged as multiple rows. Spermatheca irregular ovoid. Anterior vulval lip slightly posteriorly prolonged forming a small vulval flap. Slightly protruding posterior vulval lip. Body slightly narrowing behind

the vulva. Post-uterine branch extending two thirds to three quarters of the vulva to anus distance, acting as a seminal receptacle. Tail with a distinct mucron of 2-5 µm length with finely rounded terminus or tapering like a pencil (digitate-uniformly conoid).

Male. Anterior body region and cuticle similar to that of female. Posterior body bent ventrally when killed by heat. Testis outstretched, occupying about two thirds of the body length. Spermatocytes arranged in multiple rows. Tail ventrally arcuate with a pointed, talon-like terminus bearing a distinct, usually two-pointed terminal bursa of variable shape, which can be seen in dorso-ventral position (Fig.1 I). Spicules paired, small and slightly arcuate with pointed rostrum. Distal ends of spicules with small cucullus. Condylus high and strongly dorsally bent (Fig.1 H). There are two pairs of ventro-lateral caudal papillae (one adanal and one in the middle of the tail). The single mid-ventral papilla before the anus that occurs in most other *Bursaphelenchus* species was not observed. The position of the papillae is shown in Figs. 1-3.

Table 1. Measurements of *Bursaphelenchus yongensis* sp. n. Measurements in µm, mean±s.d. (range).

	Females		Males	
	Holotype	Paratype	Allotype	Paratype
n		15		15
L	952.0	927.1±57.2 (816.0-1,023.0)	746.0	819.2±66.4 (699.0-926.0)
a	44.5	42.1±2.3 (37.0-45.1)	54.5	57.2±5.7 (48.6-70.5)
b	12.6	11.8±1.1 (9.7-13.2)	9.3	10.9±1.1 (8.8-12.7)
c	28.6	29.8±2.3 (25.3-33.1)	27.7	30.7±3.6 (23.7-37.7)
c'	3.1	3.1±0.3 (2.6-3.7)	2.2	2.3±0.3 (1.8-2.9)
V	74.2	73.9±1.5 (68.9-75.8)	–	–
post-uterine sac	152.5	132.9±16.8 (119.5-187.8)	–	–
stylet	14.7	14.1±0.8 (12.6-15.7)	13.0	13.2±0.8 (11.7-14.6)
spicules as arc	–	–	11.5	12.4±0.6 (11.5-13.6)

Diagnosis and relationships. *Bursaphelenchus yongensis* sp. n. possesses a body length of 927 (816-1023) µm and 746 (699-926) µm for females and males, respectively, a slim body (a = 42 and 57, respectively) tapering anteriorly as well as posteriorly, 13-14 µm long stylet, excretory pore at level of median bulb, lateral field with three lines, long post-uterine branch extending two thirds to three quarters of the vulva to anus distance and a conoid female tail (c = 30) showing usually a 2-5 µm long mucron in central position at the terminus, male with small spicules (12-14 µm long) having distinct pointed rostrum, high distinctly dorsally bent condylus and small cucullus, and a dorso-ventrally visible terminal bursa of variable shape, but often two-pointed.

Bursaphelenchus yongensis sp. n. is morphologically similar to *B. eremus*. Measurements, spicules shape and shape of female tail terminus are similar. However, in the original description of *B. eremus* by Rühm (1956), important characters, such as the number of lateral lines, the shape of median bulb and the position of the excretory pore, were omitted. *B. yongensis* sp. n. differs from *B. eremus* by the presence of a cucullus at the distal end of the spicules, the differently formed spicula condylus being higher and more strongly dorsally bent than in *B. eremus*, the position of the second pair of caudal papillae being more anterior than in *B. eremus*, the differently formed bursa, the presence of a small vulval flap and the longer mucron of females.

Corresponding shapes of the spicule and the female tail of *Bursaphelenchus yongensis* sp. n. resemble those of *B. maxbassiensis* (Massey, 1971) Baujard, 1989, which also possesses a vulval flap, a long post-uterine sac and three lateral lines. This latter species has, in contrast to *B. yongensis* sp. n., an exceedingly long stylet (23-26 µm) with prominent knobs, a differently shaped lip region with an angular overhang and a heavily sclerotized tail terminus of females (Massey, 1971). Additionally, the new species shows a more strongly dorsally bent condylus of the spicules.

Two other three-lined *Bursaphelenchus* species, *B. leoni* and *B. silvestris* (Lieutier & Laumond, 1978) Baujard, 1980, have spicules that always have a strongly dorsally-bent condylus, the head separated from the body by a very distinct constriction, a short vulval flap and a two-pointed bursa. However, the females of these species have a long attenuated tail (c = 9-15) compared to the digitate tail of *B. yongensis* sp. n. (c = 30). The species of the '*leoni*' group (Braasch, 2001) have the high a value (around 40) in common with the new species, whereas most of the three-lined species of the '*hofmanni*' group within the genus *Bursaphelenchus* (Braasch, 2001) have a lower a value with the exception of *B. pinasteri*. However, the spicules of *B. pinasteri* and *B. hofmanni* have no dorsally-bent condylus, and the females show a differently formed tail with a distinct narrowing behind the anus in *B. pinasteri* and a conoid and curved tail with rounded terminus in *B. hofmanni*.

Compared to the four-lined species, the females

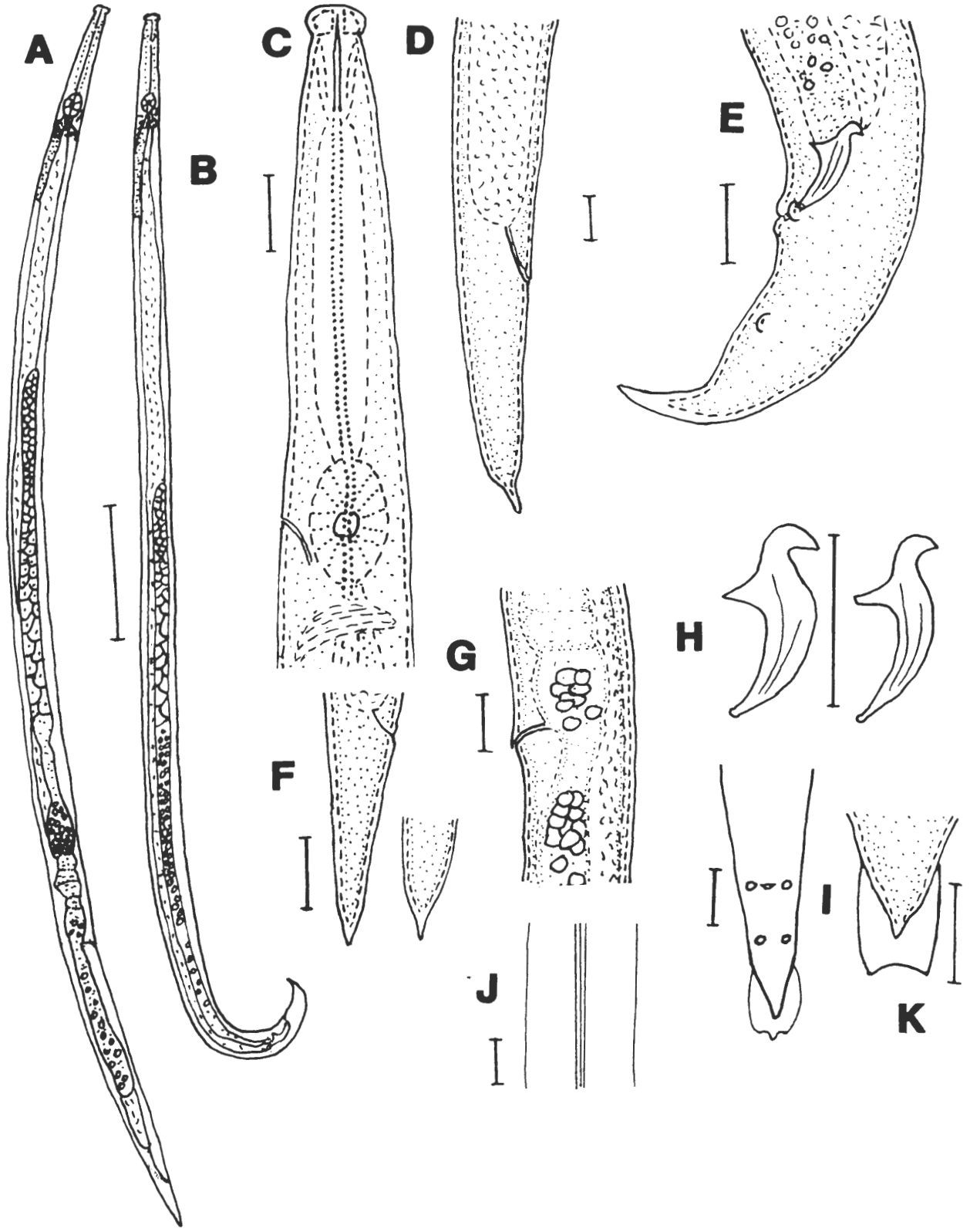

Fig. 1. *Bursaphelenchus yongensis* sp. n. A: female; B: male; C: anterior body; D: female tail; E: male tail; F: variation of female tail; G: vulval region; H: spicules; I: bursa; J: lateral field, scale bars=10 μm.

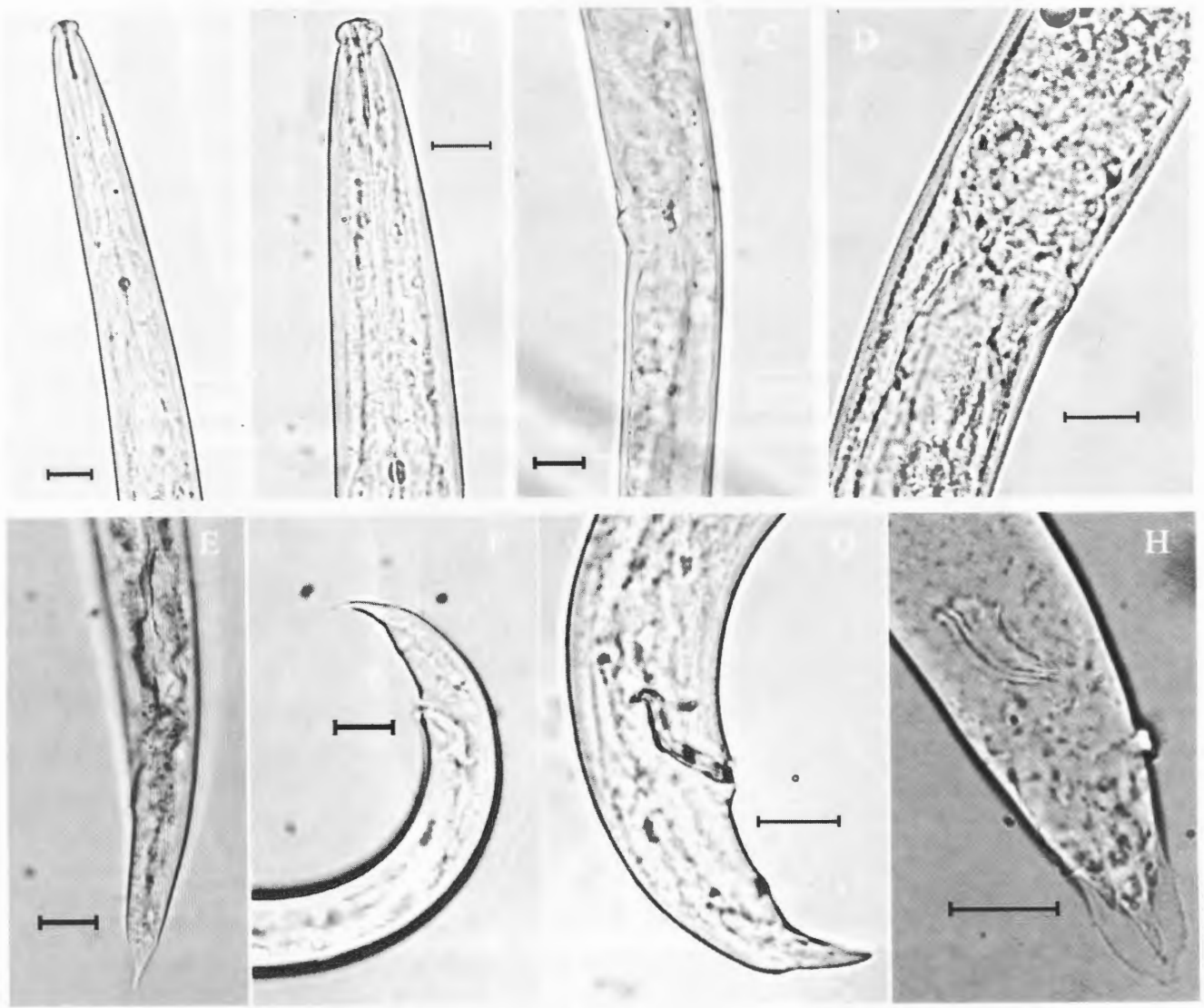

Fig. 2. Light photomicrographs of *Bursaphelenchus yongensis* sp. n. A: head region; B: female tail; C & E: male tail; D :vulval region; F: bursa, scale bars=10 μm.

of *B. yongensis* sp. n. resemble the females of *B. mucronatus* Mamiya & Enda, 1979, *B. fraudulentus* Rühm, 1956 and the mucronate forms of *B. xylophilus* with respect to their size, the slim body, the mucron at the tail terminus, the presence of a vulval flap and the narrowing of the body behind the vulva. However, the vulval flap of *B. yongensis* sp. n. is, much shorter than those of all mucronate species or forms of the '*xylophilus*' group, the mucron has a central position (often ventral in *B. mucronatus*), and the shape of spicules is totally different from the species of the '*xylophilus*' group, which have strongly recurved spicules.

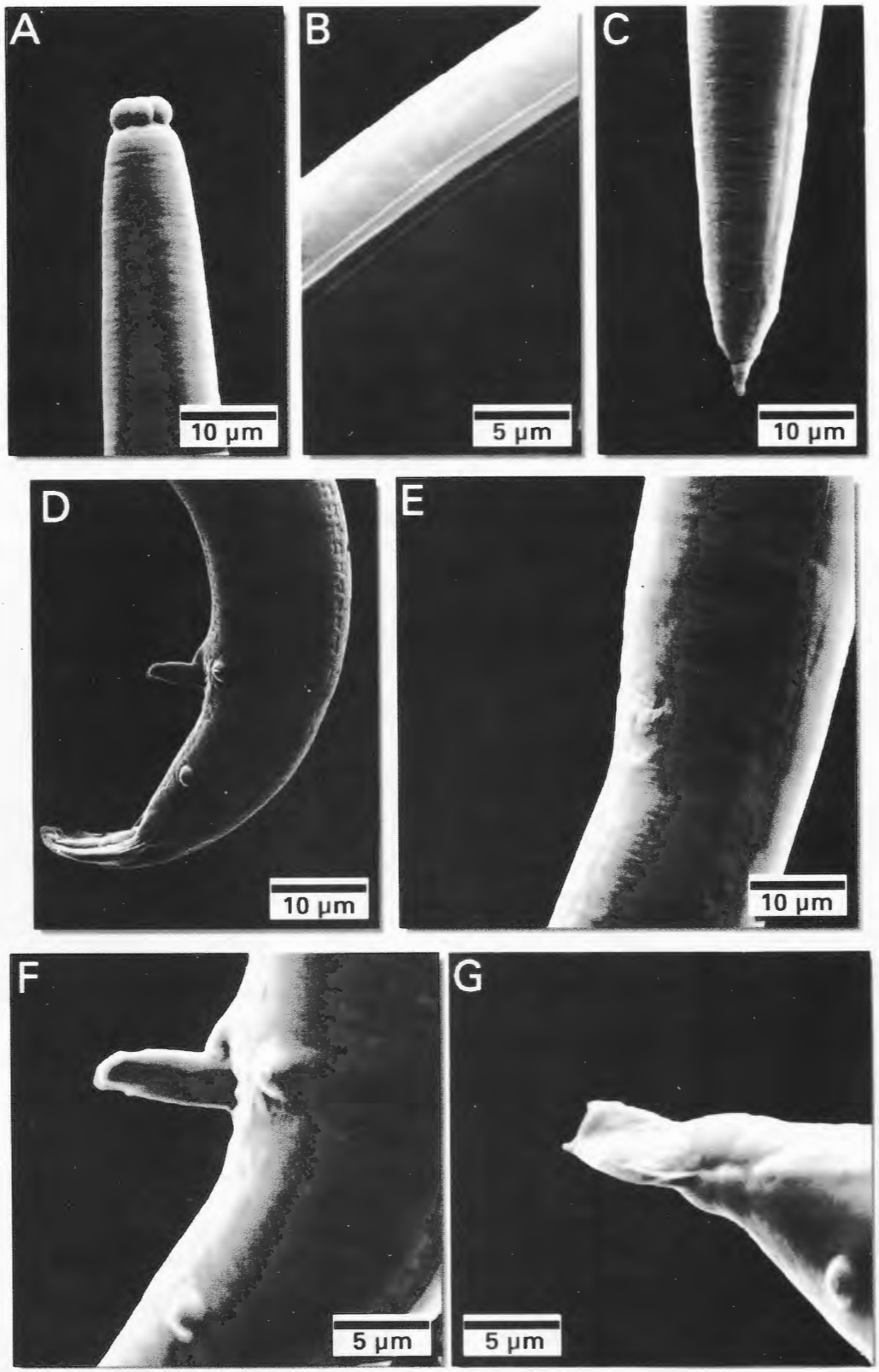

Fig. 3. Scanning electron microscope (SEM) observations of *Bursaphelenchus yongensis* sp.n. A: head region; B: lateral field; C: female tail; D: male tail; E: vulva; F: details of male tail showing spicule tip (cucullus) and papillae; G: bursa.

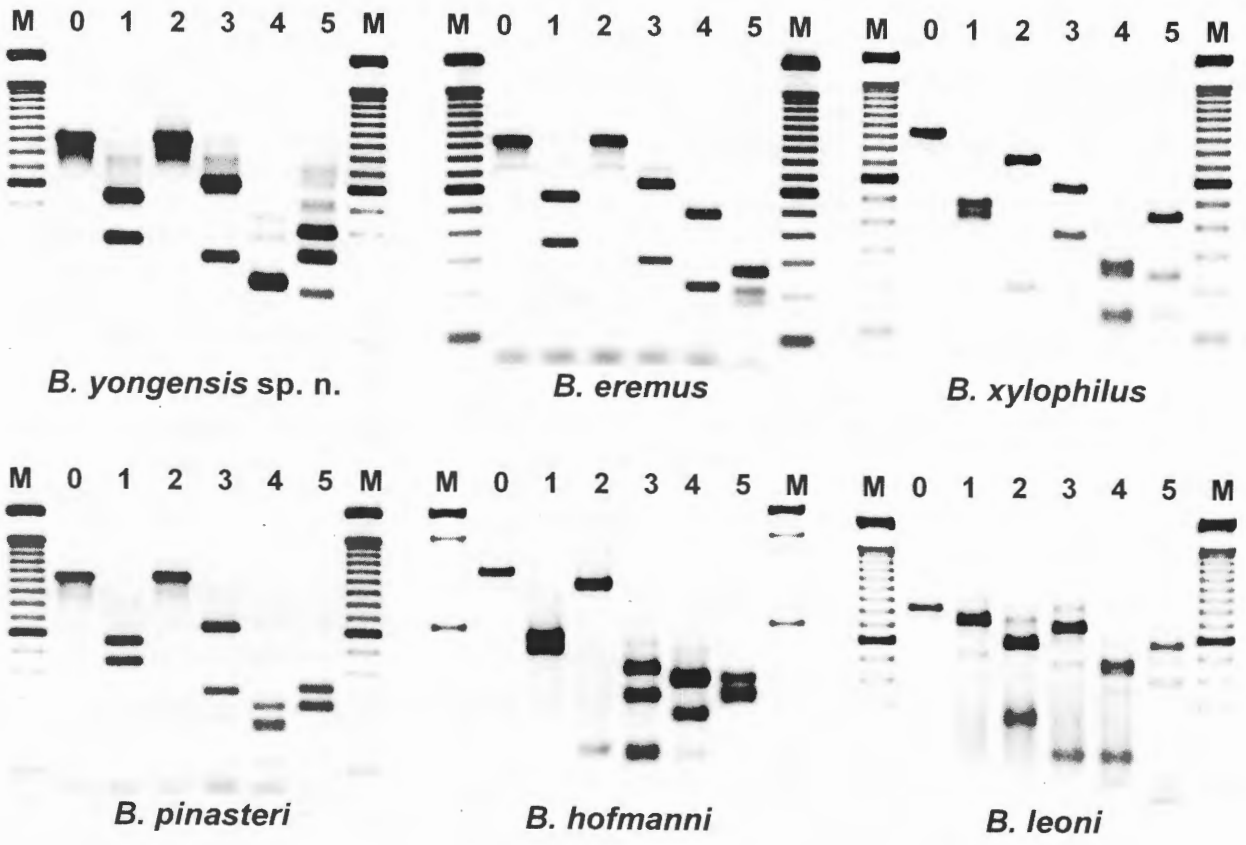

Fig. 4. ITS-RFLP patterns of *Bursaphelenchus yongensis* sp. n., the similar species *B. eremus* and four additional *Bursaphelenchus* species. Restriction fragments were obtained by digestion of the amplified rDNA fragment (0) with *Rsa*I (1), *Hae*III (2), *Msp*I (3), *Hinf*I (4) and *Alu*I(5). M: DNA marker (100 bp ladder, Invitrogen Life Technologies).

Molecular differentiation of *B. yongensis* sp. n. from other *Bursaphelenchus* species.

Amplification of the ITS-1/2 region of rDNA of *B. yongensis* sp. n. resulted in a PCR product of approximately 950 bp (Fig. 4, Tab. 2). The same size of amplicon was observed with *B. eremus, B. xylophilus* (Fig. 4, Tab. 2), as well as *B. mucronatus*, *B. luxuriosae* Kanzaki & Futai, 2003, *B. tusciae* Ambrogioni & Palmisano, 1998 and *B. seani* Giblin & Kaya, 1983 (Burgermeister *et al.*, 2005). In contrast, different amplicon sizes were obtained with the morphologically similar species *B. pinasteri*, *B. hofmanni* (1050 bp) and *B. leoni* (850 bp). The ITS-RFLP pattern of *B. yongensis* sp. n. is different from the patterns of the other species shown in Fig. 4. It also differs from the ITS-RFLP patterns of 22 additional *Bursaphelenchus* species established in earlier investigations (Burgermeister *et al.*, 2005).

As seen in Fig. 4 and Tab. 2, the PCR product of *B. yongensis* sp. n. and *B. eremus* was not cleaved by *Hae*III and produced very similar fragment patterns on digestion with *Rsa*I and *Msp*I. With both of these enzymes, the small size difference of 20 to 40 bp in the larger restriction fragment may be explained by additional restriction sites near the end of this fragment in *B. yongensis* sp. n. In conclusion, the results of ITS-RFLP analysis support a close genetic relationship between *B. yongensis* sp. n. and *B. eremus* as suggested from the morphological similarity of these species.

Type locality and habitat. *B. yongensis* sp. n. was isolated in August 2004 by the first author (G. J.) from dying *Pinus massoniana* in Xiaogang, Ningbo (synonym Yong), China.

Types. Collected from a culture on *Botrytis cinerea* on malt agar. Slides are deposited in the nematode collection of Ningbo Entry-exit Inspection and Quarantine Bureau, China and in the German Nematode Collection at the Federal Biological Research Centre for Agriculture and Forestry, Institute for Nematology and Vertebrate Research, Toppheideweg 88, 48161 Münster, Germany. A culture is available at the

Department for Plant Health of the Federal Biological Research Centre for Agriculture and Forestry, Messeweg 11/12, 38104 Braunschweig, Germany.

Table 2. Approximate size of DNA fragments observed in ITS-RFLP analysis of *Bursaphelenchus yongensis* sp. n., the similar species *B. eremus* and four additional *Bursaphelenchus* species.

Species	PCR product (bp)	Restriction fragments (bp)				
		RsaI	*HaeIII*	*Msp*I	*Hinf*I	*Alu*I
B. yongensis sp. n.	950	530 360	950	620 300	220	400 310 210
B. eremus	950	570 360	950	640 300	500 220	270 220 190
B. xylophilus	950	500 420	730 200	570 380	270 260 140	460 250 140 100
B. pinasteri	1050	560 450	1050	630 340	270 220 120	340 280 130
B. hofmanni	1050	560 490	910 140	380 300 130	350 230 120	360 300 280
B. leoni	850	790	590 260	690 160	480 160 100	590 (490) 400 180

Etymology. *B. yongensis* sp. n. was named after Yong which is an alternative name of Ningbo.

Discussion. The three-lined *Bursaphelenchus* species have similar positions of their caudal papillae and, in general, one pair close to the anus and another pair on the tail in varying distances from the bursa. In addition, some species have a single papilla in front of the anus and pairs of small ventro-sublateral papillae within the bursal flap. The position of the caudal papillae of the three-lined species is distinctly different from those of the four-lined species ('*xylophilus*' group, '*sexdentati*' group and '*fungivorus*' group according to Braasch, 2001). Based on the slim bodies ('a' around 40) and the other characters mentioned, the new species appears to be close to the '*leoni*' group. DNA sequencing may be useful to elucidate the evolutionary relationship among these species and species group.

The morphologically very similar *B. eremus* was isolated from its vector *Scolytus intricatus* emerging from oak branches in Germany. Interestingly, the close relationship of the two species could be recognized in their ITS-RFLP patterns. However, it should be noted that *B. eremus* is an inhabitant of oaks, whereas *B. yongensis* sp. n. lives in pines. Insect attack of the sampled tree was not obvious. The vector of *B. yongensis* sp. n. remains unknown. The new species can easily be multiplied on *Botrytis cinerea* cultures on malt agar.

In addition to *B. xylophilus* and *B. mucronatus,* there are two other large and slim *Bursaphelenchus* species (*B. lini, B. yongensis* sp. n.) in dying pines in China. This raises questions about the means of their differentiation from the pathogenic *B. xylophilus.* Morphological differentiation is possible for an experienced nematologist using high microscopic magnification and observing the shape of spicules and vulval flaps in adults, but can be difficult if only juveniles and females are available for study. However, the four species can be differentiated reliably by ITS-RFLP patterns (Burgermeister *et al.*, 2005).

Although there is no indication that either *B. lini* or *B. yongensis* sp. n. were implicated in the death of the trees in which they were found, the biology of these species still needs to be clarified.

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J. Gu, H. Braasch, W. Burgermeister, M. Brandstetter, J. Zhang. Описание *Bursaphelenchus yongensis* sp.n. (Nematoda: Parasitaphelenchidae), выделенного из *Pinus massoniana* в Китае.

Резюме. Описан *Bursaphelenchus yongensis* sp. n. выделенный в Нинбо (Китай) из *Pinus massoniana*. Длина тела и индекс 'a' самок и самцов 927 (816 - 1023) мкм и 746 (699 - 926) мкм и 42 и 57 мкм, соответственно. Стилет длиной 13-14 мкм, экскреторная пора на уровне медианного бульбуса, латеральное поле с тремя линиями. Длина поствульварного мешка - 60-75% от расстояния вульва-анус. Хвост самок конический (c = 30), обычно с мукро длиной 2-5 мкм. Спикулы небольшие (длина 12-14 мкм) с отчетливым заостренным рострумом, дорсально загнутым кондилюсом и небольшим кукулюсом. Терминальная бурса изменчивой формы. *Bursaphelenchus yongensis* sp. n. отличается от близкого *B. eremus* наличием кукуллюса, формой спикул, более сильно загнутым кондилюсом, а также наличием небольшой вульварной складки и более длинным мукро у самок. Новый вид также дифференцируется от 26 других видов *Bursaphelenchus* по особенностям ITS-RFLP.